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Sox9 Induction, Ectopic Paneth Cells, and Mitotic Spindle Axis Defects in Mouse Colon Adenomatous Epithelium Arising From Conditional Biallelic *Apc* Inactivation

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We generated transgenic mice in which human *CDX2* gene elements control expression of a tamoxifen-regulated Cre protein (*CDX2P-CreER^{T2}*) to allow for inducible gene targeting in intestinal epithelium. After tamoxifen dosing of *CDX2P-CreER^{T2}* mice, Cre activity was detected in the distal ileal, cecal, colonic, and rectal epithelium, with selected crypt base, transit amplifying, and surface cells all capable of activating Cre function. Four weeks after tamoxifen dosing of *CDX2P-CreER^{T2}* mice carrying a Cre-activated fluorescent reporter, single crypts were uniformly fluorescence positive or negative, reflecting Cre activation in crypt stem cells. Biallelic inactivation of the *Apc* tumor suppressor gene via the *CDX2P-CreER^{T2}* transgene in colon epithelium led to acute alterations in cell proliferation, apoptosis, and morphology, along with mitotic spindle misorientation, β-catenin nuclear localization, and induction of the intestinal stem cell markers Lgr5 and Musashi-1 and the Sox9 transcription factor. Normal mouse colon epithelium lacks Paneth cells, a key small intestine niche cell type, and Paneth cell differentiation is dependent on Sox9 function. In *Apc*-deficient colon epithelium, ectopic Paneth-like cells were seen outside the crypt base, such as new crypt budding sites. Our data indicate *Apc* inactivation via *CDX2P-CreER^{T2}* targeting in mouse colon epithelium is sufficient to induce adenomatous changes and the generation of Paneth-like cells from neoplastic progenitors, with potentially significant roles in colon adenoma development and progression. *(Am J Pathol 2013, 183: 493–503; http://dx.doi.org/10.1016/j.ajpath.2013.04.013)*

Colorectal cancer (CRC), like other cancers, manifests accumulated defects in pathways regulating cell proliferation, differentiation, and death. The defects include alterations that lead to novel or increased function of oncogenes and loss of function of tumor suppressor genes.[1] A few genes, such as the *APC*, *KRAS*, *TP53,* and *PIK3CA* genes, are each frequently mutated in CRCs and/or adenomas, but many other genes are found to be mutated only in subsets of CRCs.[1] A recent continuum model suggests how gene defects may relate to tumor location in the intestinal tract.[2] Recurrent gene defects with key roles in tumor initiation, progression, and/or maintenance are often termed *drivers*, whereas rare or unique gene alterations in cancers of a given type may represent *passenger* defects that arose coincident with a driver alteration. Sorting out driver and passenger alterations in CRC might be aided by work with genetically engineered mouse models to assess the functional significance of particular gene defects in tumorigenesis. In addition, many CRCs arise from adenomatous precursors, and adenoma development and progression to CRC may be significantly affected by interactions between colon epithelial cells with genetic and epigenetic defects and the innate and cellular immune responses to intestinal microbial communities and products.[3]

Germline *APC* gene mutations underlie the familial adenomatous polyposis syndrome, in which hundreds to thousands of adenomas arise in the colon and rectum of affected individuals in early adult life.[4] Approximately 80% of sporadic adenomas and CRCs have somatic inactivating

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mutations, leading to premature truncation of the APC protein; the somatic biallelic *APC* mutations appear to be early and likely rate-limiting events in neoplastic colon tumors.[1,4] The APC protein functions in regulating the levels and localization of the free pool of β-catenin protein.[5] Among other possible effects, APC inactivation may mimic activation of β-catenin in response to Wnt ligands, with β-catenin accumulating in the cytoplasm and nucleus and binding to T-cell factor (TCF) family transcription factors, in turn, activating expression of varied TCF-regulated genes.[5] Gene expression profiling studies suggest the transcriptional program induced by β-catenin stabilization in CRCs may resemble the transcriptional program in normal intestinal and colon crypt base columnar stem cells (CBCSCs).[5,6] Further work has also implicated β-catenin activation in the spatial organization and migration of cells in crypts.[7,8] The consequences in small intestinal epithelium after biallelic inactivation of Apc have been addressed,[8,9] but the acute effects of Apc inactivation on colon epithelium are not well understood.

Mouse Cdx2 and human CDX2 proteins are homeodomain-containing transcription factors, and *Cdx2/CDX2* expression is normally largely restricted to small intestine and colon epithelial cells in the adult.[10,11] We previously described *CDX2P-NLSCre* transgenic mice in which 9.5 kb of human *CDX2* regulatory sequences were used to regulate expression of Cre recombinase in caudal tissues during development and in epithelium of the distal ileum, cecum, colon, and rectum during adult life.[12] By using the *CDX2P-NLSCre* transgene to inactivate one *Apc* allele somatically, we generated a mouse model of colonic adenoma-carcinoma progression. We also previously described *CDX2P-G22Cre* transgenic mice with mosaic Cre expression in a subset of colorectal crypts,[13] and we used the *CDX2P-G22Cre* transgenic mice to study phenotypic consequences in colon epithelium after biallelic, inactivating mutations in *Apc* or oncogenic gain-of-function mutations in *Kras*.[14] Transgenic mice expressing ligand [tamoxifen (TAM)]-regulated Cre recombinase—so-called CreER^{T2} [a fusion protein containing Cre recombinase sequences fused to a mutated ligand-binding domain of human estrogen receptor 1 protein that responds to TAM and not endogenous estrogen (ER^{T2})]—in colon epithelium might allow for studies of the effects of targeting a wide array of genes in adult colon epithelium. We describe the generation of *CDX2P-CreER^{T2}* transgenic mice. We used reporter lines with Cre-activated transgenes [β-galactosidase (β-gal)[15] and enhanced yellow fluorescent protein (EYFP)[16]] to monitor functional Cre induction in response to TAM treatment. We also report in detail on the temporal sequence of events after Cre-mediated inactivation of *Apc* in colon crypts, including the generation of ectopic Paneth-like cells in *Apc*-deficient colon epithelium at positions outside the crypt base, such as where new crypt budding occurs. Given findings implicating Paneth cells as a key supporting cell type in the stem cell niche in normal intestinal crypts,[17] the data imply a potentially significant role for Paneth cells in neoplastic progression.

Materials and Methods

Plasmids

The 9.5-kb fragment containing 5′-flanking sequence from the human *CDX2* promoter region has previously been described.[18] To assemble the *CDX2P-CreER^{T2}* transgene construct, both *CDX2* promoter and a CreER^{T2} DNA fragment were assembled into the pcDNA3 vector (Invitrogen, San Diego, CA), which provides a polyadenylation cassette from the bovine growth hormone gene (*BGHpA*).

Mice

CDX2P-CreER^{T2} transgenic founders on a mixed background [C57BL6/J (B6) × SJL/J] were back-crossed with B6 mice. To monitor Cre expression, male F2 mice from the transgenic line were intercrossed with mice carrying the *Gt(ROSA)26Sor^{tm1Sor}/J* reporter allele (R26R)[15] or the *Gt(ROSA)26Sor $^{tm1(EYFP)Cos}$/J* reporter allele (EYFP)[16] (The Jackson Laboratory, Bar Harbor, ME). As a comparison, we crossed *Lgr5-EGFP-IRES-CreER^{T2}* (B6.129P2-*Lgr5$^{tm1(cre/ERT2)Cle}$*/J) mice[19] with *Gt(ROSA)26Sor^{tm1Sor}/J* reporter mice. *Kras$^{LSL-G12D/+}$* mice have been described previously.[20] We crossed *Kras$^{LSL-G12D/+}$* mice with *CDX2P-CreER^{T2}* transgenic mice to activate the mutant *Kras* allele in the intestinal tract. To target *Apc* alleles, *CDX2P-CreER^{T2}* mice or *CDX2P-NLSCre* mice were intercrossed with mice homozygous for an *Apc* targeted allele (*Apc$^{flox/flox}$*, 580S) as described.[12,13] Animal husbandry and experimental procedures were performed with the approval of the University Committee on Use and Care of Animals, University of Michigan, and according to Michigan state and US federal regulations. All the mice were housed in specific-pathogen free conditions. After weaning, rodent 5001 chow and automatically supplied water were provided *ad libitum* to mice. The transgenic *CDX2P-CreER^{T2}* mice have been deposited with Jackson Laboratory (Jax stock No. 022390).

TAM Treatment

Mice with *CDX2P-CreER^{T2}* transgene were injected i.p. with TAM (Sigma-Aldrich, St. Louis, MO) dissolved in corn oil. For single TAM dosing, we used 200 mg/kg; for three consecutive daily doses, we administered TAM at 100 mg/kg per dose. Mice were injected with TAM at 2 to 3 months of age. Animals were euthanized and analyzed at various time points after the single injection or the final injection of three was given.

Human Tissue Specimens

Deidentified human colon tissue specimens were provided by the Department of Pathology at the University of Michigan Health System (Ann Arbor, MI).

IHC, Immunofluorescence, and β-Gal Analysis

Mouse tissues were prepared for paraffin-embedding or cryosectioning as described previously.[13] For assessment of cell proliferation, mice were pulsed with 5-bromo-2-deoxyuridine (BrdU; Sigma-Aldrich) for 1 hour before the mice were euthanized. Sections of paraffin-embedded human or mouse tissues were subjected to immunohistochemical (IHC) analysis as described.[21] The following primary antibodies were used for IHC analysis with sections of paraffin-embedded tissues: mouse anti-BrdU (1:500; BD Biosciences, San Jose, CA), rabbit anti-lysozyme (1:2000; Dako, Carpinteria, CA), and mouse anti−β-catenin (1:800; BD Biosciences). Histochemical identification of intestinal goblet cells was performed on paraffin sections with Alcian blue (Sigma-Aldrich) after IHC with lysozyme. For BrdU staining, tissue sections were treated with 2N HCl at 37°C for 30 minutes after performing antigen retrieval with citrate buffer (pH 6.0; Biogenex, San Ramon, CA). For immunofluorescence of frozen sectioned tissues, mouse colon and intestinal tissues were fixed in 4% paraformaldehyde overnight, cryoprotected, and frozen in OCT. Standard immunofluorescence staining was performed on 6-μm frozen sections. Antibodies included mouse anti−β-catenin (1:800; BD Biosciences), rabbit anti-lysozyme (1:1000; Dako), rabbit anti-chromogranin A (1:1000; ImmunoStar, Hudson, WI), goat anti−c-kit (1:200; R&D Systems, Minneapolis, MN), and goat anti-Reg4 (1:50; R&D Systems). For immunofluorescence using paraffin-embedded tissues, the following primary antibodies were used: rabbit anti-Sox9 (1:200; Millipore, Temecula, CA), rat anti−Musashi-1 (Msi1) (1:500; a gift from Dr. Hideyuki Okano[22,23]), mouse anti−α-tubulin (1:1000; Sigma-Aldrich), rabbit anti-Dclk1 (1:200; Epitomics, Burlingame, CA), and rabbit anti-Crb3 (1:1000; kindly provided by Dr. Benjamin Margolis at University of Michigan). The secondary antibodies used were Alexa fluor 488-conjugated donkey anti-goat, Alexa fluor 488-conjugated donkey anti-rabbit, Alexa fluor 488-conjugated goat anti-rabbit, Alexa fluor 594-conjugated goat anti-mouse, Alexa fluor 488-conjugated goat anti-mouse, Alexa fluor 594-conjugated goat anti-rabbit, and Alexa fluor 488-conjugated goat anti-rat (Molecular Probes, Invitrogen, Carlsbad, CA), diluted at 1:1000. DNA was labeled by Hoechst 33342 (Molecular Probes, Invitrogen) by adding to the washing buffer at 5 μg/mL. For detection of goblet cells with fluorescence, frozen sections of mouse proximal colon tissues were incubated with rhodamine *Dolichos biflorus* agglutinin (1:500 in PBS; Vector Laboratories, Burlingame, CA) at 37°C for 1 hour. β-gal analysis was performed as described previously.[13]

Mitotic Spindle Axis Assessment

The spindle angle was defined by the orientation of mitotic spindle based on α-tubulin staining, The adjacent apical membrane of the mitotic cell was defined by Crb3 staining. The mitotic spindle axis angle relative to the planar axis of the cells was then measured by ImageJ version 1.42q (National Institutes of Health, Bethesda, MD).

Statistical Analysis

All data were evaluated by Student's *t*-test, and asterisks denote significance with $P < 0.05$. Error bars denote SDs.

Results

TAM-Regulated Cre Function in Intestinal Epithelium of *CDX2P-CreER^{T2}* Mice

We generated a *CDX2P-CreER^{T2}* transgenic construct (Figure 1A) and addressed the ability of the *CDX2P-CreER^{T2}* transgene to target loxP-containing transgenes *in vivo* in murine intestinal epithelial tissues and specifically in response to TAM treatment. To assess TAM induction of Cre activity, we used two different transgenic mouse lines (R26R and EYFP) carrying integrated reporter gene sequences (ie, β-gal or EYFP, respectively) at the ubiquitously expressed Rosa26 locus.[15,16] The reporter gene products are only produced after Cre-mediated excision of a transcriptional stop element flanked by loxP sites. We bred selected *CDX2P-CreER^{T2}* transgenic founder mice to the R26R line and treated *CDX2P-CreER^{T2} R26R* progeny with TAM to activate CreER^{T2} function. To detect activation of β-gal expression after Cre-mediated targeting in the R26R line, the intestinal tract and other tissues were stained with X-gal, fixed, and analyzed by gross and microscopic analysis. We identified three transgenic lines with TAM-inducible β-gal expression in colon epithelium (data not shown) and focused here on one line (No. 752). In the absence of TAM treatment, we found no evidence of β-gal expression in mouse intestinal tissues (Supplemental Figure S1). Three daily TAM injections in 2- to 3-month-old *CDX2P-CreER^{T2} R26R* mice led to readily detectable expression of β-gal in the mucosa in the cecum and proximal colon by 3 days after the final TAM dose and in mucosa of the cecum, colon, and rectum by 2 months after the final TAM dose, along with limited and patchy β-gal staining seen in the distal ileum (Supplemental Figure S1). In contrast to the largely colonic pattern of TAM-induced Cre recombinase function in *CDX2P-CreER^{T2}* mice, at 2 months after three daily doses of TAM in adult *Lgr5-EGFP-IRES-CreER^{T2} R26R* mice,[19] Cre function was activated robustly and broadly only in the proximal third of the small intestine, with patchy and limited β-gal expression in the remainder of the small intestine, cecum, colon, and rectum (Supplemental Figure S1).

We also performed studies of Cre-mediated activation of EYFP expression from the Rosa26 locus in intestinal tissues to confirm and extend the TAM-induced β-gal results with the *CDX2P-CreER^{T2}* line 752. Three days after the last of three daily TAM doses, we studied epithelium in

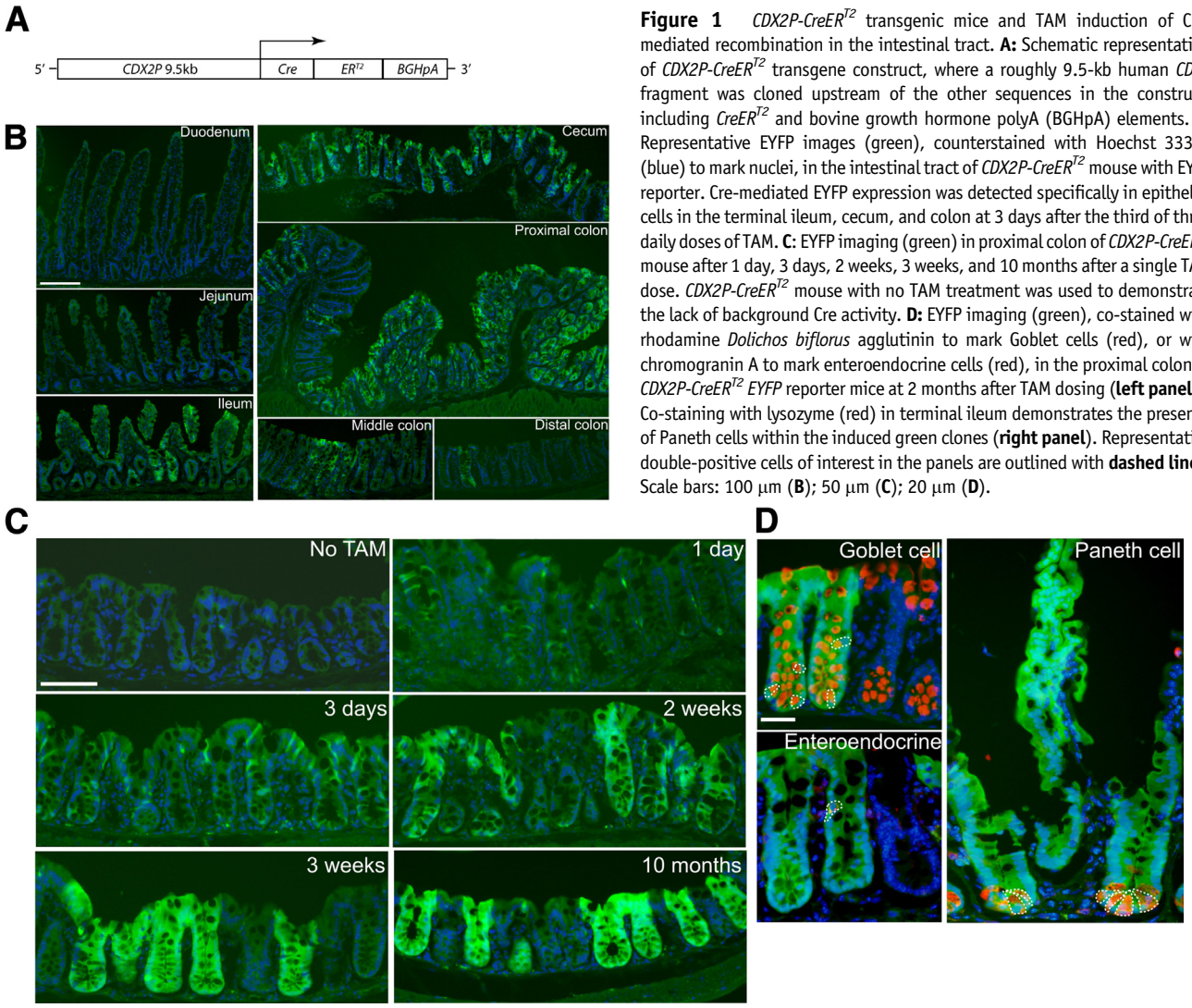

Figure 1 *CDX2P-CreER*^{T2} transgenic mice and TAM induction of Cre-mediated recombination in the intestinal tract. **A:** Schematic representation of *CDX2P-CreER*^{T2} transgene construct, where a roughly 9.5-kb human *CDX2* fragment was cloned upstream of the other sequences in the construct, including *CreER*^{T2} and bovine growth hormone polyA (BGHpA) elements. **B:** Representative EYFP images (green), counterstained with Hoechst 33342 (blue) to mark nuclei, in the intestinal tract of *CDX2P-CreER*^{T2} mouse with EYFP reporter. Cre-mediated EYFP expression was detected specifically in epithelial cells in the terminal ileum, cecum, and colon at 3 days after the third of three daily doses of TAM. **C:** EYFP imaging (green) in proximal colon of *CDX2P-CreER*^{T2} mouse after 1 day, 3 days, 2 weeks, 3 weeks, and 10 months after a single TAM dose. *CDX2P-CreER*^{T2} mouse with no TAM treatment was used to demonstrate the lack of background Cre activity. **D:** EYFP imaging (green), co-stained with rhodamine *Dolichos biflorus* agglutinin to mark Goblet cells (red), or with chromogranin A to mark enteroendocrine cells (red), in the proximal colon of *CDX2P-CreER*^{T2} *EYFP* reporter mice at 2 months after TAM dosing (**left panels**). Co-staining with lysozyme (red) in terminal ileum demonstrates the presence of Paneth cells within the induced green clones (**right panel**). Representative double-positive cells of interest in the panels are outlined with **dashed lines**. Scale bars: 100 μm (**B**); 50 μm (**C**); 20 μm (**D**).

the duodenum, jejunum, ileum, cecum, and various regions of the colon and rectum for EYFP expression, finding TAM-induced EYFP expression in epithelial cells from terminal ileum to rectum, with the strongest induction of Cre activity at early time points in the cecum and proximal colon (Figure 1B). In studies that focused on the proximal colon of 2- to 3-month-old *CDX2P-CreER*^{T2} mice treated with a single dose of TAM, we found a few scattered EYFP cells at 1 day after the TAM dose, with larger numbers of EYFP-positive cells at 3 days and 2 weeks. By 3 to 4 weeks after a single TAM dose, individual crypts were entirely EYFP positive or negative, presumably reflecting Cre-mediated activation of the EYFP reporter in crypt stem cells, which then were able to maintain EYFP-positive crypts for at least 10 months (Figure 1C). Further, consistent with the notion that TAM-induced Cre activation could occur in crypt stem cells, differentiated goblet and enteroendocrine cells in colon and Paneth cells in small intestine expressed EYFP (Figure 1D).

Biallelic *Apc* Inactivation in Colon Epithelium Acutely Alters Cell Proliferation, Death, and Expression of Wnt Pathway Genes and Stem Cell Markers

We analyzed the phenotypic effects of TAM-induced conditional inactivation of both *Apc* alleles in intestinal tissues of 2- to 3-month-old *CDX2P-CreER*^{T2} *Apc*$^{flox/flox}$ mice. At 27 days after a single TAM dose to inactivate *Apc*, we found hyperplasia of the epithelium in the terminal ileum, cecum, and colon, along with dysplastic changes such as those in adenomatous epithelium in humans and mice (Supplemental Figure S2). Time course studies indicated that within 5 days of a single TAM dose, *CDX2P-CreER*^{T2} *Apc*$^{flox/flox}$ mice had significant hyperplastic and dysplastic alterations in proximal colon epithelium (Supplemental Figure S2), indicating *Apc* inactivation is sufficient to induce adenomatous epithelium in the mouse colon. Although DNA synthesis and cell proliferation in normal mouse colon epithelium are largely restricted to the

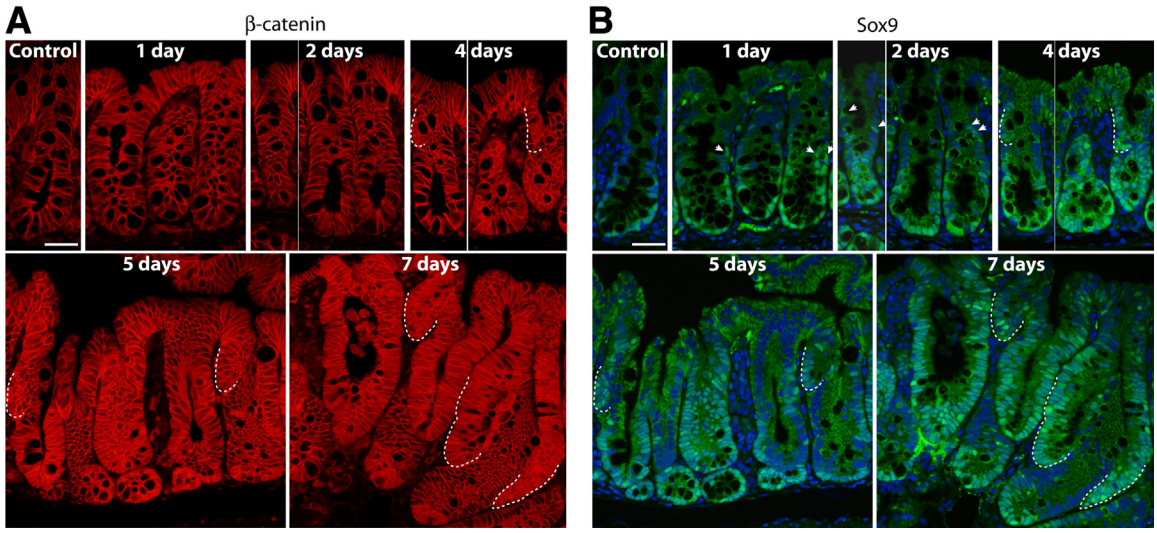

Figure 2 Conditional inactivation of *Apc* in mouse colon is linked to induction of Sox9 expression and initiation of crypt budding. Proximal colon tissues from wild-type (control) or *CDX2P-CreER^{T2} Apc$^{flox/flox}$* mice were co-stained for β-catenin (red; **A**) and Sox9 (green; **B**) and counterstained with Hoechst 33342 (blue) after 1 day, 2 days, 4 days, 5 days, and 7 days after a single TAM dose. The budded crypt regions were outlined with **dashed lines**, which indicate nuclear staining for both Sox9 and β-catenin. **Arrows** indicate the cells with increased Sox9 expression induced by inactivation of *Apc* at early time points but without β-catenin induction shown as membrane staining. Scale bar = 20 μm.

bottom third to half of each crypt, within the first 2 days after TAM dosing of *CDX2P-CreER^{T2} Apc$^{flox/flox}$* mice, we observed increased DNA synthesis in cells in the upper half of the crypt, with further increases over time (Supplemental Figure S3). Marked increases in the number of apoptotic cells were also seen in proximal colon epithelial glands by 6 days after *Apc* targeting, with further increases in the percentage of apoptotic cells over time (Supplemental Figure S3).

After a single TAM dose in 2- to 3-month-old *CDX2P-CreER^{T2} Apc$^{flox/flox}$* mice, we found increased β-catenin expression could be seen in subsets of epithelial cells from 4 days onward after the TAM dose (Figure 2A). Cells with elevated cytoplasmic and occasional nuclear β-catenin expression were more often localized near crypt base regions, including the base regions of recently branched, newly arising glands (Figure 2A). Consistent with the elevated β-catenin levels, increased expression of Sox9, a transcription factor protein encoded by a β-catenin/TCF-regulated gene,[24] was found in *Apc*-mutant (*Apc^{min}*) colon epithelium (Figure 2B). However, Sox9 induction was more readily apparent at earlier time points than changes in β-catenin expression (Figure 2B), which may reflect the ability to detect even modest changes in Sox9 expression, because of the low basal levels of Sox9 expression in normal colon epithelium. Interestingly, we also observed frequent crypt buddings (Figure 2) and adenomatous features (Figure 2 and Supplemental Figure S2) in the cells with strong β-catenin and Sox9 nuclear staining and at cell positions not restricted to the presumptive CBCSCs in normal crypts. These data indicate that *de novo* crypts during adenoma formation can perhaps be induced by *Apc* inactivation in colon crypt cells other than the presumptive CBCSCs. It is uncertain whether the ability of the *de novo* crypts arising in the colon to persist and/or progress may depend on their cell of origin, as appears to be the case when *Apc* is inactivated in the small intestine.[9] After *Apc* inactivation, we were also able to detect strong induction of enhanced green fluorescent protein (EGFP) expression from the *Lgr5* locus (Lgr5-EGFP), a β-catenin/TCF-regulated gene and a marker of presumptive CBCSCs in normal colon,[19] along with increased expression of Msi1 RNA-binding protein, another presumptive stem cell marker[25,26] (Figure 3). A recent study has suggested that the Dclk1 protein might be a marker to distinguish between tumor stem cells and normal stem cells in the intestine and specifically that Dclk1 marks potential tumor stem cells in *Apc^{min}* small intestine tumors.[27] Although Dclk1 expression was seen in one to three cells in each normal colon crypt, Dclk1-expressing cells were rarely seen in *Apc*-defective colon crypts (Supplemental Figure S4).

Apc Inactivation Rapidly Generates Ectopic Paneth Cells in Colon Epithelium

Paneth cells, a specialized cell type intercalated with CBCSCs in the normal small intestine, have been proposed to play a critical functional role in maintenance of the crypt stem cell niche, perhaps via secretion of trophic factors for CBCSCs, such as Wnts, Notch, and epidermal growth factor receptor ligands.[17] Sox9 function is required for Paneth cell differentiation,[28,29] and Apc inactivation in small intestine epithelium has been found to generate increased numbers of lysozyme-expressing Paneth-like cells in the resultant adenomatous epithelium.[8,30,31] Normal mouse colon epithelium lacks Paneth cells. However, within 4 days after TAM dosing of 2- to 3-month-old *CDX2P-CreER^{T2} Apc$^{flox/flox}$* mice, we found lysozyme-expressing Paneth-like cells in the targeted colon epithelium (Figure 4). Lysozyme-expressing

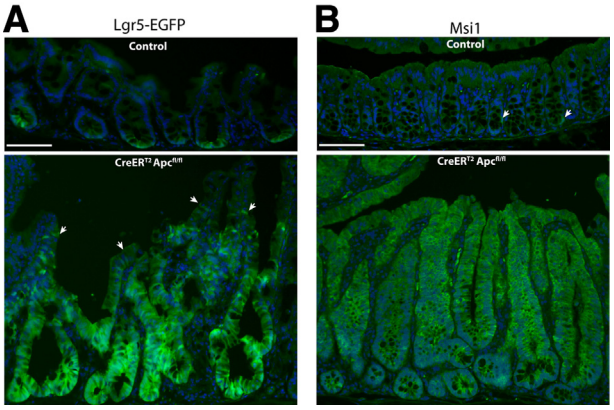

Figure 3 Biallelic *Apc* inactivation in mouse colon epithelium induces intestinal stem cell marker expression. **A:** EGFP imaging (green), counterstained with Hoechst 33342 (blue) to mark nuclei, in the proximal colon of the *CDX2P-CreER^{T2}*, *Lgr5-EGFP-IRES-CreER^{T2}*, *Apc$^{flox/flox}$* mouse or the control *Lgr5-EGFP-IRES-CreER^{T2}* mouse at 20 days after TAM dosing. **Arrows** indicate the cells positive for Lgr5 at a region close to the luminal surface in mouse with *Apc* inactivation. **B:** Immunofluorescence staining for Msi1 (green) and counterstained with Hoechst 33342 (blue) in the proximal colon of wild-type (control) or *CDX2P-CreER^{T2} Apc$^{flox/flox}$* mice at 21 days after TAM induction. **Arrows** indicate the cells positive for Msi1 at the crypt base in normal colon epithelium. Scale bar = 50 μm.

cells were preferentially located in crypt regions with strong β-catenin expression, including sometimes near the base of crypts undergoing branching or fission (Figure 4), implying that the Paneth-like cells in *Apc*-mutant colon epithelium might contribute to a conducive environment, perhaps akin to a niche, for potential adenoma stem cells outside the normal crypt base. Crypt branching, a potential key early event in small intestinal and colon polyp formation, was described as the formation of an outpocketing pouch in a single crypt in mice carrying a truncation mutation at codon 716 of *Apc* (ApcΔ716).[32]

In normal mouse small intestinal epithelium, lysozyme-expressing Paneth cells are restricted to the crypt base and lack expression of markers for other intestinal cell lineages, such as the Alcian blue staining that marks goblet cells with intestinal-type mucin expression (Figure 5A). In *Apc*-mutant mouse colon epithelium, although there was a notable decrease in Alcian blue staining, reflecting loss of differentiated goblet cells, a few Alcian blue-staining cells were seen and a subset of the Alcian blue-positive cells also expressed lysozyme (Figure 5A). Thus, the data suggest *Apc* inactivation had altered the colon epithelial differentiation program to generate possible bipotential precursor cells that could differentiate into goblet and/or Paneth cell fates. Studies of human colon adenomas demonstrated lysozyme-expressing Paneth-like cells were also frequent in the lesions studied and a subset of the lysozyme-expressing adenoma cells also stained with Alcian blue (Figure 5B). After Apc inactivation in colon epithelium of 2- to 3-month-old *CDX2P-CreER^{T2} Apc$^{flox/flox}$* mice, although many of the lysozyme-expressing Apc-mutant cells were not actively synthesizing DNA, a subset of the lysozyme-expressing colon epithelial cells did in fact reveal BrdU incorporation (Figure 5C). This result suggests that these lysozyme-expressing cells might not only have an altered differentiation program but also have an altered proliferation potential. To further evaluate the differentiation status of lysozyme-expressing colon epithelial cells, we analyzed the expression of two putative markers of crypt base secretory cells.[17,33] Most of the lysozyme-expressing colon epithelial cells also expressed the crypt base secretory cell marker known as regenerating islet-derived family member-4 (Reg4),[17,33] but these cells represented only a fraction of Reg4-positive cells (Figure 5D). c-Kit has been suggested as a marker of potential crypt base secretory cells that support Lgr5^{+} colon stem cells in the mouse small intestine.[33] Although c-Kit expression was seen in the normal mouse colon crypt base region, the lysozyme-expressing *Apc*-mutant colon epithelial cells lacked c-Kit expression (Figure 5D).

Apc-Defective Mouse Colon Crypts Acutely Manifest Spindle Axis Abnormalities

The orientation of the mitotic spindle is believed to have a critical role in regulating cell fate decisions in the intestine and other tissues because the cytokinetic cleavage plane that generates two daughter cells is oriented perpendicular to the

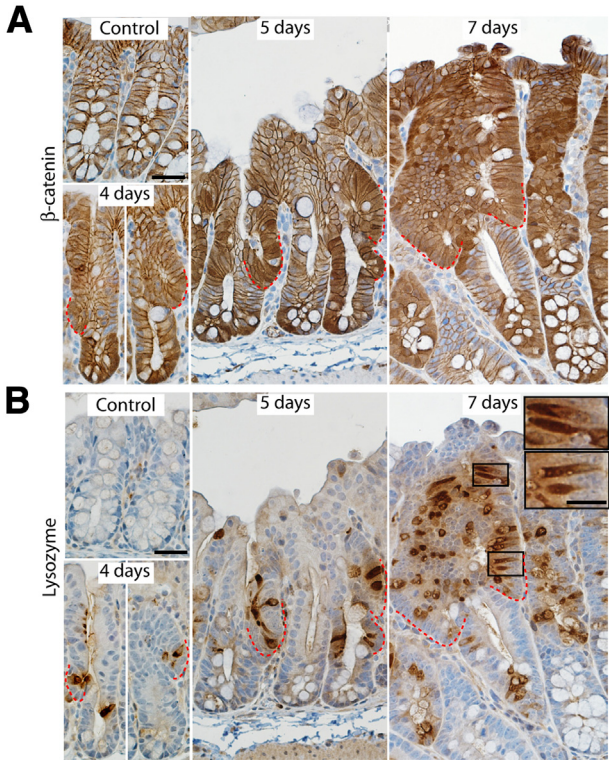

Figure 4 Inactivation of *Apc*, β-catenin expression, and crypt budding associated with altered cell differentiation in mouse colon. IHC staining for β-catenin (**A**) and lysozyme (**B**) in the proximal colon of wild-type (control) or *CDX2P-CreER^{T2} Apc$^{flox/flox}$* mice at 4 days, 5 days, and 7 days after a single TAM induction. **Insets:** High magnifications for the boxed area. Scale bars: 20 μm (low magnifications); 10 μm (**insets**, high magnifications).

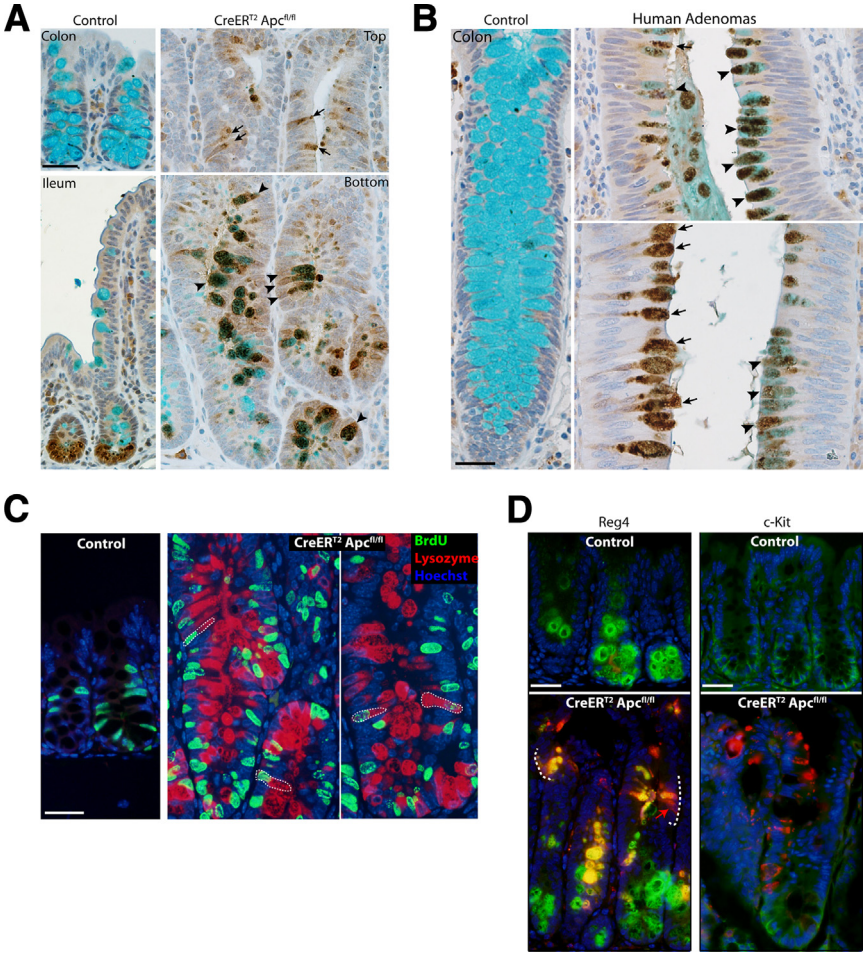

Figure 5 Characteristics of lysozyme-expressing cells in *Apc*-defective colon epithelium. **A:** IHC staining for lysozyme, followed by Alcian blue staining, in the proximal colon (**left upper panel**) and ileum (**left bottom panel**) from a wild-type mouse (control) or proximal colon from a *CDX2P-CreER^{T2} Apc$^{flox/flox}$* mouse (**right panel**) at 21 days after TAM dosing. The crypt base (**bottom right panel**) and a region close to the luminal surface (**top right panel**) are shown. **B:** IHC staining for lysozyme, followed by Alcian blue staining, in control human colon mucosa (**left panel**) and two human adenomas (**right panel**). **Arrowheads** indicate the double-positive cells for lysozyme and Alcian blue staining; **arrows** indicate the cells only positive for lysozyme. **C:** Proximal colon tissues from wild-type (control) or TAM-induced (for 7 days) *CDX2P-CreER^{T2} Apc$^{flox/flox}$* (**right panel**) mice were co-stained for BrdU (green) and lysozyme (red) and counterstained with Hoechst 33342 (blue). The double positive cells were outlined with **dashed lines. D:** Proximal colon tissues from wild-type (control) or TAM-induced (for 7 days) *CDX2P-CreER^{T2} Apc$^{flox/flox}$* (**bottom panel**) mice were stained for Reg4 (**left panel**, green) or c-Kit (**right panel**, green), co-stained with lysozyme (red), and counterstained with Hoechst 33342 (blue). The budded crypts were outlined with **dashed lines**. Scale bar = 20 μm.

spindle axis.[34,35] In a tissue that has a single columnar cell layer, such as colon epithelium, if the spindle axis is oriented in a planar fashion (ie, parallel to the crypt axis), cell division generates two daughter cells with essentially equivalent luminal (apical) and basement (extracellular matrix) surfaces. If the spindle axis is not oriented parallel to the crypt axis, cell division generates daughter cells with differences in luminal and basement membrane surfaces, perhaps resulting in differential exposures of the daughter cells to extracellular cues and/or unequal segregation of cytoplasmic determinants, with the potential for resultant differences in the cell fates adopted by the two daughter cells.[34,35] Prior work has indicated that *Apc* heterozygous mutant and homozygous intestinal epithelial cells have mitotic spindle axis orientation defects.[36–38]

We have assessed spindle axis orientation in mitotic cells independent of their position in the crypt in colon epithelial tissues from wild-type mice, as well as mice with heterozygous *Apc* somatic defects (*CDX2P-NLSCre Apc$^{flox/+}$* mice) and 2- to 3-month-old *CDX2P-CreER^{T2} Apc$^{flox/flox}$* mice at 21 days after a single TAM dose to inactivate both *Apc* alleles. We also studied mitotic spindle orientation in hyperplastic and hyperproliferative colon epithelium of mice where a Cre recombinase-dependent *Kras* mutant oncogenic allele[20] was activated by TAM-induced activation of the *CDXP-CreER^{T2}* transgene. Most mitoses evaluated in all mouse tissues were outside the crypt base region. In >75% of mitotic cells from normal mouse colon epithelium and *Apc* heterozygous-mutant and *Kras*-mutant colon epithelium, mitotic spindles were aligned within 30° of the planar (crypt) axis, but only approximately 50% of *Apc* homozygous mutant epithelial cells had mitotic spindle axes within the 30° of the planar axis, with roughly 30% of *Apc* homozygous mutant cells having spindle axes from 30° to 60° out of planar alignment and nearly 20% showing spindle axes between 60° and 90° out of planar alignment (Figure 6, A and B). Similar to the situation in the mouse tissues, we found that human colon adenomas, most of which harbor biallelic *APC* mutations, had upwards of 50% of the mitotic cells with spindle axis orientation ≥30° degrees out of the planar axis, whereas >75% of the mitotic epithelial cells in normal colon and human hyperplastic polyps with *KRAS*-activating mutations had their spindle axes within 30° of the planar axis (Figure 6, C and D).

Discussion

Several genetically engineered mouse models permitting inducible Cre recombinase targeting of loxP-containing

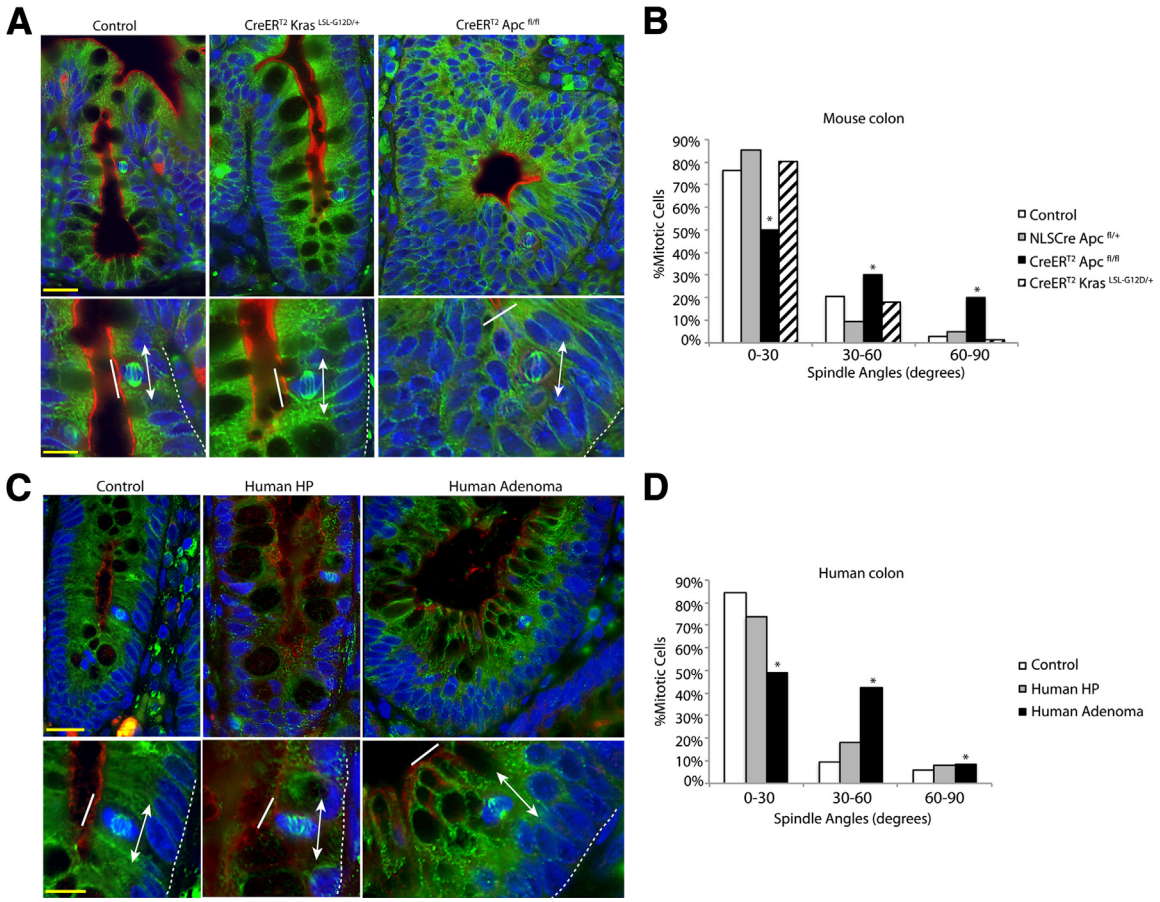

Figure 6 Misalignment of mitotic spindle axes with the planar axis in mouse and human colon adenomatous epithelium but not in hyperplastic *Kras*-mutant mouse colon epithelium or human hyperplastic polyps (HP). **A:** Proximal colon tissues from wild-type (control) or *CDX2P-CreER^{T2} Kras$^{LSL-G12D/+}$* (**middle panel**) mice or *CDX2P-CreER^{T2} Apc$^{flox/flox}$* (**right panel**) mice at 21 days after TAM dosing were co-stained for α-tubulin (green) and Crb3 (red) and counterstained with Hoechst 33342 (blue). Representative low- (**upper panel**) or high-magnification (**lower panel**) images are shown. **B:** The spindle angles are defined by the orientation of mitotic spindles versus the most adjacent apical membrane indicated by Crb3 staining. Quantification for the indicated tissues is shown, including colon tissues from *CDX2P-NLSCre Apc$^{flox/+}$* (heterozygous) mice. **C:** Tissues from normal human colon, human hyperplastic polyps, or human adenomas were co-stained for α-tubulin (green) and Crb3 (red) and counterstained with Hoechst 33342 (blue). Representative low- (**upper panel**) or high-magnification (**lower panel**) images are shown. **A** and **C: Double-headed arrows** indicate the orientation of mitotic spindles. **Solid lines** indicate the most adjacent apical membrane of the mitotic cell, which is used as a reference for the spindle angle. **Dashed lines** indicate the border of the basement membrane. **D:** Quantification for panel **C** was shown. *$P < 0.01$ compared with the normal tissues ($n = 3$ and >100 crypts were counted). Scale bars: 20 μm (**A** and **C**, low magnifications); 10 μm (**A** and **C**, high magnifications). *$P < 0.01$ compared with the normal tissues ($n = 3$ and >100 crypts were counted).

genes in intestinal tissues have been described, such as *Villin-CreER^{T2}*, *AhCre*, and *Lgr5-EGFP-IRES-CreER^{T2}* transgenic mice.[19,39,40] All three of these transgenic lines have proven valuable for targeting genes of interest in intestinal tissues, such as the *Apc* and *Ctnnb1* genes and various other genes implicated in tumorigenesis and/or cell fate specification. Nonetheless, because the Villin promoter elements are quite broadly active throughout the intestinal tract and the *Lgr5* and *Ah (Cyp1A)* genes are also expressed in tissues other than epithelium of the intestinal tract, there are challenges in using the respective Cre recombinase transgenes to assess the physiologic consequences specifically in the colon epithelium for genes of interest. In our prior work, we described the use of upstream regulatory elements of the human *CDX2* gene (*CDX2P*) for directing transgene expression to most epithelial cells of the very distal ileum, cecum, colon, and rectum during adult life.[12] We have described here the generation and characterization of *CDX2P-CreER^{T2}* transgenic mice, including the utility of the *CDX2P-CreER^{T2}* transgene for TAM-dependent targeting of reporter genes in colon epithelium and for studies of ligand-regulated targeting of other genes of interest, such as the *Apc* tumor suppressor gene.

Prior studies of colon epithelium from patients with familial adenomatous polyposis demonstrated clear-cut changes in cell proliferation patterns in flat colonic mucosa, with focal and widespread loss of the normal repression of DNA synthesis from the upper portion of the crypt in the colon mucosa of those with familial adenomatous polyposis.[41,42] Most published studies of the physiologic consequences of biallelic inactivation of the mouse *Apc* gene have emphasized effects seen in small intestine epithelium. Among the phenotypic effects reported in intestinal epithelium after *Apc* biallelic inactivation were the following:

increased proliferation; increased apoptosis; decreased cell migration along the crypt-villus axis; and altered differentiation, including loss of villus markers and goblet cells and the generation of increased numbers of Paneth cells, especially at ectopic positions outside the crypt base.[8,30] Only a few publications have reported in any depth on selected effects of inactivating *Apc* or modulating β-catenin function in mouse colon epithelium, such as the article by Andreu et al,[31] indicating that *Apc* inactivation led to the apparent *de novo* specification of Paneth cells in colon epithelium. Also, a recent article from Hirata et al[43] reported that activation of a stabilized mutant form of β-catenin had variable effects on cell phenotypes, depending on the levels of β-catenin protein expression achieved in colon epithelium of adult mice, including increased number of $Lgr5^+$ cells, increased crypt fission rates in colon epithelium, and *de novo* crypt formation.

In our studies, we established that multiple effects seen after *Apc* biallelic inactivation in small intestine epithelium are also seen when *Apc* is inactivated in colon epithelium, including the following: increased cell proliferation, increased apoptosis, decreased number of goblet cells, and the generation of ectopic Paneth cells. We also found that induction of certain β-catenin/TCF-regulated genes, such as *Sox9*, was even more easily detectable than β-catenin stabilization at early time points after *Apc* inactivation in the colon epithelium. Also, stem cell marker gene expression, such as that for the Wnt pathway-regulated *Lgr5* gene and for *Msi1*, which both normally mark presumptive CBCSCs, could be seen in a large fraction of the *Apc*-defective colon epithelial cells, independent of cell position within a crypt. Of some interest, although the *Dclk1* gene has recently been suggested to be a marker for cancer stem cells in small intestine tumors arising in Apc^{min} mutant mice and not a marker of normal intestinal stem cells,[27] we found very rare Dclk1-expressing cells in *Apc*-defective mouse colon epithelium, raising some questions about the proposal that Dclk1 expression will be a key marker for colon cancer stem cells in human.

The rapid induction of Sox9 protein expression in *Apc*-defective colon epithelium is potentially noteworthy, given not only that *Sox9* gene expression is regulated by Wnt/β-catenin/TCF signaling but also that the Sox9 protein appears to function as a negative feedback regulator of β-catenin function in the nucleus in some settings.[24,28] Perhaps more significantly, Sox9 has been implicated as a key factor for the differentiation of Paneth cells in small intestine epithelium.[28,29] The potent induction of Sox9 in *Apc/APC*-mutant colon epithelium may be a contributing factor to the generation of the lysozyme-expressing Paneth-like cells seen in mouse and human adenomatous tissues in the colon, including in our studies. We have chosen to term the various lysozyme-expressing cells that we observed in our studies as Paneth-like because a subset of the ectopically generated cells seem to express both lysozyme, characteristic of Paneth cells, and intestinal mucin, characteristic of goblet cells. In addition, some of the lysozyme-expressing *Apc*-defective mouse colon epithelial cells incorporated BrdU, whereas mature Paneth cells are thought to be postmitotic. Interestingly, prior work has demonstrated the induction of ectopic expression of Paneth cell makers in intestinal villus and colon crypts in mice maintained on a Western-style diet, which can induce the development of small and large intestine tumors in the mice after long-term exposure.[44] Although dietary-induced changes in gene expression profiles were distinct from those in mice with genetic risk of intestinal tumors due to a constitutional *Apc* mutation,[44] mice with nutritional or genetic risk may share some common features, such as ectopic expression of Paneth-like cells, suggesting some overlapping mechanisms in tumor development.

Although it was initially suggested that Paneth cells were an essential cell type in the stem cell niche supporting CBCSCs,[17] several recent studies suggest that Paneth cells may not be absolutely required for development and maintenance of CBCSCs *in vivo*.[14,28,29,45,46] It has also been suggested that Paneth cells are not required for the initiation of *Apc* mutation-induced adenomatous epithelium in the small intestine in the mouse.[45] Nevertheless, given the likely important trophic role of Paneth cell surface and secreted factors on CBCSCs, including perhaps Notch ligands, R-spondins, and epidermal growth factor receptor ligands, it is possible that the Paneth-like cells generated by the neoplastic progenitors in *Apc*-mutant colon epithelium may play important contributing roles in adenoma formation and progression. Consistent with this suggestion, we find that the Paneth-like cells present at early time points after *Apc* inactivation were not infrequently associated with stabilized β-catenin staining patterns and new crypt branching sites. In our study, a fraction of lysozyme-expressing cells had indications of altered proliferation and differentiation properties, suggesting that these Paneth-like cells might have multilineage potential or be induced to reenter the cell cycle to produce more such cells as a support for tumor growth. Two recent studies have suggested a key role for a subset of intestinal crypt cells expressing Paneth cell markers as the potential reserve cells for the more rapidly cycling Lgr5-positive CBCSCs in the setting of intestinal homeostasis or injury.[47,48] Further studies will be needed to clarify the possible functional contribution of the Paneth-like cells present in *Apc/APC*-mutant colon epithelium and human adenomas. The Paneth-like cells could function as potential reserve stem cells, sources of growth factor ligands, and/or in other yet to be identified ways in tumor development and progression.

Besides the changes in proliferation and loss of normal differentiation patterns that developed very rapidly after *Apc* inactivation in murine colon epithelium, we observed the rapid acquisition of nuclear stratification and other changes in cell morphology and polarity akin to those seen in adenomatous epithelium in human and mouse. The means by which *Apc* inactivation contributes to these complex changes in cell phenotype are not well understood. In previously published work, it has been suggested that even nonneoplastic *Apc* heterozygous mutant epithelium may demonstrate changes in mitotic spindle axis orientation, including in

presumptive CBCSCs.[35–38] In our studies, we focused on the marked changes from the typical planar spindle polarity present in most mitotic normal colon epithelial cells that were seen in *Apc/APC*-mutant colon epithelium of mice and humans, respectively. Specifically, although only a small fraction of normal mitotic spindle axes were oriented $\geq 30^{\circ}$ from the planar axis, roughly 50% of *Apc/APC*-mutant epithelium was $>30^{\circ}$ out of alignment with the planar axis. Spindle misorientation might in fact be a key contributing feature to dysplasia and other cytologic features characteristic of adenomatous epithelium and its propensity to be at risk for neoplastic progression. In contrast, spindle misorientation might be lacking in cells and tissues without neoplastic features or with a low likelihood of progression to neoplastic features, such as hyperplastic epithelium.[35] Consistent with this view, we found that *Kras/KRAS*-mutant epithelium in hyperplastic lesions in mice and humans had mitotic spindle polarity akin to that in normal colon epithelium. Interestingly, in contrast to some previous studies,[36–38] we have not seen the defect in mitotic spindle axis orientation in nondysplastic *Apc* heterozygous mutant epithelium in our *CDX2P-NLSCre* $Apc^{flox/+}$ mouse model. This difference may be because heterozygous inactivation of *Apc* by the *CDX2P-NLSCre* transgene only occurs in intestine and colon epithelium at later stages of development or in adult tissues, whereas *Apc* is constitutionally defective in both colon epithelium and stromal cells in the Apc^{min} mouse used in previous studies. In addition, the difference in results may reflect our focus on the proximal colon epithelium compared with the small intestine or distal colon in the work on Apc^{min} mouse tissues.

In closing, we have described the generation and characterization of *CDX2P-CreER*^{T2} transgenic mice, a new transgenic model that should prove to be very valuable for studies of the physiologic functions of selected genes in colon epithelium *in vivo*. We have found that biallelic inactivation of *Apc* leads to many acute changes in proliferation, apoptosis, and morphology, along with mitotic spindle axis misorientation, β-catenin nuclear localization, and induction of stem cell marker genes and the Sox9 transcription factor. The robust induction of Sox9 in *Apc*-defective cells may be contributing to the generation of ectopic Paneth-like cells in adenomatous colon epithelium in humans and mice, and the Paneth-like cells may play potentially important roles in neoplastic crypt maintenance and progression. Future studies with the *CDX2P-CreER*^{T2} transgenic mice are likely to address various uncertainties about function of selected genes in colon epithelium, such as the individual and collective effects of oncogene and tumor suppressor gene defects in CRC development. In addition, the *CDX2P-CreER*^{T2} transgenic mouse model may provide an important research tool for assessing the potentially complex factors and interactions contributing to colorectal tumor development, including how dietary factors and the intestinal microenvironment and immune system may intersect with genetic and epigenetic factors in tumor initiation and progression.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.04.013*.

References

1. Fearon ER: Molecular genetics of colorectal cancer. Annu Rev Pathol 2011, 6:479–507
2. Yamauchi M, Morikawa T, Kuchiba A, Imamura Y, Qian ZR, Nishihara R, Liao X, Waldron L, Hoshida Y, Huttenhower C, Chan AT, Giovannucci E, Fuchs C, Ogino S: Assessment of colorectal cancer molecular features along bowel subsites challenges the conception of distinct dichotomy of proximal versus distal colorectum. Gut 2012, 61:847–854
3. Grivennikov SI, Wang K, Mucida D, Stewart CA, Schnabl B, Jauch D, Taniguchi K, Yu GY, Osterreicher CH, Hung KE, Datz C, Feng Y, Fearon ER, Oukka M, Tessarollo L, Coppola V, Yarovinsky F, Cheroutre H, Eckmann L, Trinchieri G, Karin M: Adenoma-linked barrier defects and microbial products drive IL-23/IL-17-mediated tumour growth. Nature 2012, 491:254–258
4. Rustgi AK: The genetics of hereditary colon cancer. Genes Dev 2007, 21:2525–2538
5. Clevers H, Nusse R: Wnt/beta-catenin signaling and disease. Cell 2012, 149:1192–1205
6. van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP, Tjon-Pon-Fong M, Moerer P, van den Born M, Soete G, Pals S, Eilers M, Medema R, Clevers H: The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002, 111:241–250
7. Batlle E, Henderson JT, Beghtel H, van den Born MM, Sancho E, Huls G, Meeldijk J, Robertson J, van de Wetering M, Pawson T, Clevers H: Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell 2002, 111:251–263
8. Sansom OJ, Reed KR, Hayes AJ, Ireland H, Brinkmann H, Newton IP, Batlle E, Simon-Assmann P, Clevers H, Nathke IS, Clarke AR, Winton DJ: Loss of Apc in vivo immediately perturbs Wnt signaling, differentiation, and migration. Genes Dev 2004, 18:1385–1390
9. Barker N, Ridgway RA, van Es JH, van de Wetering M, Begthel H, van den Born M, Danenberg E, Clarke AR, Sansom OJ, Clevers H: Crypt stem cells as the cells-of-origin of intestinal cancer. Nature 2009, 457:608–611
10. Beck F: The role of Cdx genes in the mammalian gut. Gut 2004, 53: 1394–1396
11. Saad RS, Ghorab Z, Khalifa MA, Xu M: CDX2 as a marker for intestinal differentiation: its utility and limitations. World J Gastrointest Surg 2011, 3:159–166
12. Hinoi T, Akyol A, Theisen BK, Ferguson DO, Greenson JK, Williams BO, Cho KR, Fearon ER: Mouse model of colonic

adenoma-carcinoma progression based on somatic Apc inactivation. Cancer Res 2007, 67:9721–9730
13. Akyol A, Hinoi T, Feng Y, Bommer GT, Glaser TM, Fearon ER: Generating somatic mosaicism with a Cre recombinase-microsatellite sequence transgene. Nat Methods 2008, 5:231–233
14. Feng Y, Bommer GT, Zhao J, Green M, Sands E, Zhai Y, Brown K, Burberry A, Cho KR, Fearon ER: Mutant KRAS promotes hyperplasia and alters differentiation in the colon epithelium but does not expand the presumptive stem cell pool. Gastroenterology 2011, 141: 1003–1013. e1001–e1010
15. Soriano P: Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet 1999, 21:70–71
16. Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F: Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol 2001, 1:4
17. Sato T, van Es JH, Snippert HJ, Stange DE, Vries RG, van den Born M, Barker N, Shroyer NF, van de Wetering M, Clevers H: Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 2011, 469:415–418
18. Hinoi T, Loda M, Fearon ER: Silencing of CDX2 expression in colon cancer via a dominant repression pathway. J Biol Chem 2003, 278: 44608–44616
19. Barker N, van Es JH, Kuipers J, Kujala P, van den Born M, Cozijnsen M, Haegebarth A, Korving J, Begthel H, Peters PJ, Clevers H: Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 2007, 449:1003–1007
20. Jackson EL, Willis N, Mercer K, Bronson RT, Crowley D, Montoya R, Jacks T, Tuveson DA: Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. Genes Dev 2001, 15:3243–3248
21. Feng Y, Bommer GT, Zhai Y, Akyol A, Hinoi T, Winer I, Lin HV, Cadigan KM, Cho KR, Fearon ER: Drosophila split ends homologue SHARP functions as a positive regulator of Wnt/beta-catenin/T-cell factor signaling in neoplastic transformation. Cancer Res 2007, 67:482–491
22. Kaneko Y, Sakakibara S, Imai T, Suzuki A, Nakamura Y, Sawamoto K, Ogawa Y, Toyama Y, Miyata T, Okano H: Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. Dev Neurosci 2000, 22:139–153
23. Sakakibara S, Imai T, Hamaguchi K, Okabe M, Aruga J, Nakajima K, Yasutomi D, Nagata T, Kurihara Y, Uesugi S, Miyata T, Ogawa M, Mikoshiba K, Okano H: Mouse-Musashi-1, a neural RNA-binding protein highly enriched in the mammalian CNS stem cell. Dev Biol 1996, 176:230–242
24. Blache P, van de Wetering M, Duluc I, Domon C, Berta P, Freund JN, Clevers H, Jay P: SOX9 is an intestine crypt transcription factor, is regulated by the Wnt pathway, and represses the CDX2 and MUC2 genes. J Cell Biol 2004, 166:37–47
25. Nishimura S, Wakabayashi N, Toyoda K, Kashima K, Mitsufuji S: Expression of Musashi-1 in human normal colon crypt cells: a possible stem cell marker of human colon epithelium. Dig Dis Sci 2003, 48:1523–1529
26. Potten CS, Booth C, Tudor GL, Booth D, Brady G, Hurley P, Ashton G, Clarke R, Sakakibara S, Okano H: Identification of a putative intestinal stem cell and early lineage marker; musashi-1. Differentiation 2003, 71:28–41
27. Nakanishi Y, Seno H, Fukuoka A, Ueo T, Yamaga Y, Maruno T, Nakanishi N, Kanda K, Komekado H, Kawada M, Isomura A, Kawada K, Sakai Y, Yanagita M, Kageyama R, Kawaguchi Y, Taketo MM, Yonehara S, Chiba T: Dclk1 distinguishes between tumor and normal stem cells in the intestine. Nat Genet 2013, 45:98–103
28. Bastide P, Darido C, Pannequin J, Kist R, Robine S, Marty-Double C, Bibeau F, Scherer G, Joubert D, Hollande F, Blache P, Jay P: Sox9 regulates cell proliferation and is required for Paneth cell differentiation in the intestinal epithelium. J Cell Biol 2007, 178:635–648
29. Mori-Akiyama Y, van den Born M, van Es JH, Hamilton SR, Adams HP, Zhang J, Clevers H, de Crombrugghe B: SOX9 is required for the differentiation of Paneth cells in the intestinal epithelium. Gastroenterology 2007, 133:539–546
30. Andreu P, Colnot S, Godard C, Gad S, Chafey P, Niwa-Kawakita M, Laurent-Puig P, Kahn A, Robine S, Perret C, Romagnolo B: Crypt-restricted proliferation and commitment to the Paneth cell lineage following Apc loss in the mouse intestine. Development 2005, 132:1443–1451
31. Andreu P, Peignon G, Slomianny C, Taketo MM, Colnot S, Robine S, Lamarque D, Laurent-Puig P, Perret C, Romagnolo B: A genetic study of the role of the Wnt/beta-catenin signalling in Paneth cell differentiation. Dev Biol 2008, 324:288–296
32. Oshima H, Oshima M, Kobayashi M, Tsutsumi M, Taketo MM: Morphological and molecular processes of polyp formation in Apc (delta716) knockout mice. Cancer Res 1997, 57:1644–1649
33. Rothenberg ME, Nusse Y, Kalisky T, Lee JJ, Dalerba P, Scheeren F, Lobo N, Kulkarni S, Sim S, Qian D, Beachy PA, Pasricha PJ, Quake SR, Clarke MF: Identification of a cKit(+) colonic crypt base secretory cell that supports Lgr5(+) stem cells in mice. Gastroenterology 2012, 142:1195–1205. e1196
34. Graham TA, Jawad N, Wright NA: Spindles losing their bearings: does disruption of orientation in stem cells predict the onset of cancer? Bioessays 2010, 32:468–472
35. Pease JC, Tirnauer JS: Mitotic spindle misorientation in cancer–out of alignment and into the fire. J Cell Sci 2011, 124:1007–1016
36. Caldwell CM, Green RA, Kaplan KB: APC mutations lead to cytokinetic failures in vitro and tetraploid genotypes in Min mice. J Cell Biol 2007, 178:1109–1120
37. Fleming ES, Temchin M, Wu Q, Maggio-Price L, Tirnauer JS: Spindle misorientation in tumors from APC(min/+) mice. Mol Carcinog 2009, 48:592–598
38. Quyn AJ, Appleton PL, Carey FA, Steele RJ, Barker N, Clevers H, Ridgway RA, Sansom OJ, Nathke IS: Spindle orientation bias in gut epithelial stem cell compartments is lost in precancerous tissue. Cell Stem Cell 2010, 6:175–181
39. el Marjou F, Janssen KP, Chang BH, Li M, Hindie V, Chan L, Louvard D, Chambon P, Metzger D, Robine S: Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis 2004, 39:186–193
40. Ireland H, Houghton C, Howard L, Winton DJ: Cellular inheritance of a Cre-activated reporter gene to determine Paneth cell longevity in the murine small intestine. Dev Dyn 2005, 233:1332–1336
41. Deschner EE, Lipkin M: Proliferative patterns in colonic mucosa in familial polyposis. Cancer 1975, 35:413–418
42. Lipkin M, Blattner WE, Fraumeni JF Jr, Lynch HT, Deschner E, Winawer S: Tritiated thymidine (phi p, phi h) labeling distribution as a marker for hereditary predisposition to colon cancer. Cancer Res 1983, 43:1899–1904
43. Hirata A, Utikal J, Yamashita S, Aoki H, Watanabe A, Yamamoto T, Okano H, Bardeesy N, Kunisada T, Ushijima T, Hara A, Jaenisch R, Hochedlinger K, Yamada Y: Dose-dependent roles for canonical Wnt signalling in de novo crypt formation and cell cycle properties of the colonic epithelium. Development 2013, 140:66–75
44. Wang D, Peregrina K, Dhima E, Lin EY, Mariadason JM, Augenlicht LH: Paneth cell marker expression in intestinal villi and colon crypts characterizes dietary induced risk for mouse sporadic intestinal cancer. Proc Natl Acad Sci U S A 2011, 108:10272–10277
45. Durand A, Donahue B, Peignon G, Letourneur F, Cagnard N, Slomianny C, Perret C, Shroyer NF, Romagnolo B: Functional intestinal stem cells after Paneth cell ablation induced by the loss of transcription factor Math1 (Atoh1). Proc Natl Acad Sci U S A 2012, 109: 8965–8970
46. Garabedian EM, Roberts LJ, McNevin MS, Gordon JI: Examining the role of Paneth cells in the small intestine by lineage ablation in transgenic mice. J Biol Chem 1997, 272:23729–23740
47. Buczacki SJ, Zecchini HI, Nicholson AM, Russell R, Vermeulen L, Kemp R, Winton DJ: Intestinal label-retaining cells are secretory precursors expressing Lgr5. Nature 2013, 495:65–69
48. Roth S, Franken P, Sacchetti A, Kremer A, Anderson K, Sansom O, Fodde R: Paneth cells in intestinal homeostasis and tissue injury. PLoS One 2012, 7:e38965